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Gene Defects Clustered at the C-Terminus of the vpr Gene of HIV-1 in Long-Term Nonprogressing Mother and Child Pair: *In Vivo* Evolution of vpr Quasispecies in Blood and Plasma

BIN WANG,* YING CHUN GE,* PAMELA PALASANTHIRAN,† SHI-HUA XIANG,‡ JOHN ZIEGLER,† DOMINIC E. DWYER,* CHRISTINE RANDLE,* DAVID DOWTON,* ANTHONY CUNNINGHAM,* and NITIN K. SAKSENA*,[1]

*Retroviral Genetics Laboratory, Department of Virology, Westmead Hospital, ICPMR, Westmead, New South Wales, 2145, Sydney, Australia; †Department of Pediatric Immunology, Prince of Wales Childrens Hospital, Randwick, New South Wales, 2013, Australia; and ‡Storr Liver Unit, Department of Medicine, University of Sydney, Westmead Hospital, Westmead, New South Wales 2145, Sydney, Australia

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Earlier studies on HIV-1 strains from HIV-1-infected long-term nonprogressors (LTNP) have reported that nef deletions and/or attenuations may be crucial in the survival of these patients. Other reports have suggested that the nef gene may not be the only gene involved, but attenuations in other accessory genes (vif, vpr, vpu), which play an important role in the viral life cycle, may be similarly important in chronic HIV-1 infection in LTNPs. Here we show the molecular and phylogenetic analyses of the vpr gene in HIV-1 strains derived from both blood and plasma of an HIV-1 infected long-surviving mother–child pair which has survived for >13 years with HIV infection: both have maintained stable $CD4^+$ T-cell counts. Analyses of blood- and plasma-derived HIV-1 vpr clones indicated the presence of defects (insertions and deletions) and length polymorphisms. Interestingly, all the vpr defects in PBMCs and plasma were clustered at the C-terminus of the Vpr protein, between amino acid residues 83 and 89, which has been implicated in the G2 cell cycle arrest as a step to early HIV-1 infection. In contrast, the vpr sequence analysis of HIV-1 strains derived from 30 different patients, who either died of AIDS-related illnesses or have AIDS, showed neither C-terminal defects nor length polymorphism in the vpr gene. Also, secondary structure predictions suggest that the naturally occurring mutations at the C-terminal region (aa 83–89) have the potential to affect the secondary structure of the Vpr protein. Also, in some cases, the out-of-frame mutations and the length polymorphisms affect the tat gene reading frame. Together, these mutations may have potential significance in conferring chronic HIV-1 infection in this long-surviving nonprogressing mother–child pair. © 1996 Academic Press, Inc.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) (*1*). Experimental evidence from simian and human immunodeficiency virus (SIV and HIV), respectively, has suggested that attenuations in the nef gene of HIV-1 derived from infected long-term survivors or nonprogressors (LTNPs) are critical in providing protection *in vivo* (*2, 3–9*). In addition, recent data suggest that attenuations in other accessory genes such as vpr, vif, rev, and tat may also be participating in providing natural protection to HIV-1-infected LTNPs (*8–10*). The Vpr protein, which is primarily localized in the nucleus of infected cells (*11, 12*), can be immunogenic with 30–40% of HIV-1-infected individuals, demonstrating anti-Vpr antibodies (*13*). Recently, the mutated vpr gene has been suggested in readily establishing long-term chronic infection of T cells, whereas normal Vpr is known to increase the rate of viral replication and cytopathic effects of the virus in cell culture (*14, 15*). Furthermore, expression of Vpr alone may affect the progression of cells in the cell cycle (*16*). In an interesting finding Weiner and co-investigators (*11*) suggested that extracellular Vpr protein can increase cellular permissiveness to HIV-1 replication and reactivate virus from latency. The same group also showed that serum Vpr could also activate virus expression from resting PBMCs of HIV-1-infected individuals. Thus, Vpr may have the potential to participate in the activation of replication and controlling latency of HIV-1 *in vivo* (*17*).

According to several recent reports (*18, 19*), Vpr is involved in G2 cell cycle arrest and inducing cytoskeletal changes as a step to early HIV-1 infection. Recently, it has also been demonstrated that the N-terminal domain of Vpr is involved in the nuclear localization (*20, 21*) and the incorporation of Vpr into HIV-1 virions (*22, 16*), whereas the C-terminus is crucial in G2 cell cycle arrest (*18*). Therefore, it is likely that mutations at the C-terminus

[1] To whom correspondence and reprint requests should be addressed. Fax: 61-2 (639 7431). E-mail: Nitins@Westmed.WH.SU.EDU.AU.

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may have the potential to alter and/or mute the G2 phase arresting signal, which could lead to chronic HIV infection.

Although the mechanisms that allow for long-term survival in HIV-1-infected LTNPs are not understood, it is likely that controlled viral replication of HIV-1 strains in LTNPs and the hosts' immunological make-up are involved (*23–31*). In this context, the role of viral gene attenuations in conferring protection cannot be underestimated; it is likely that the attenuations are partially responsible for rendering the virus noninfectious by slowing the replication. Given the above functions and also the role of the C-terminal region of Vpr in G2 cell cycle arrest, Vpr is an attractive candidate for studying its role in LTNPs or chronic HIV-1 infection.

In an attempt to elucidate the molecular mechanisms for long-term survival in HIV-1-infected individuals, we have dissected the quasispecies of the vpr gene of HIV-1 derived from PBMCs and plasma of the first mother (LW) and child (JW) pair which has survived for >13 years with HIV-1 infection. They have no apparent sign of AIDS-related illness and have maintained stable CD4+ T-cell counts (700 and 750/μl blood, respectively) from 1983 to 1995. The mother was infected via blood transfusion, while pregnant, and in 1983 was confirmed positive by ELISA. At the end of 1983, she had a baby boy who was uninfected at the time of birth. In 1984, the baby contracted the HIV-1 infection via breast feeding and tested positive for anti-HIV-1 antibodies. The PBMC samples available to us were from 1987, 1989, 1992, 1993, 1994, and 1995, whereas the plasma samples available were only from 1994 and 1995. This report is novel with respect to its first description of a HIV-1-infected long-surviving mother–child pair. Here we describe a detailed picture of HIV-1 vpr quasispecies evolution in PBMCs (collected in 1987, 1989, 1992, 1993, 1994, and 1995) and in plasma (collected in 1994 and 1995), along with the secondary-structure analysis of Vpr protein and its possible implications on chronic HIV-1 infection.

Patients' blood was collected into acid citrate tubes and was subjected to Ficoll–Hypaque gradient centrifugation at 1000 *g* for 10 min within 3 hr of collection. Peripheral blood mononuclear cells were separated, and the plasma was further subjected to centrifugation for 20 min in order to eliminate any residual cells. These fresh PBMCs from both mother (LW) and child (JW) were used in virus co-culture studies. For these, we employed 5×10^6 fresh PBMCs from patients LW and JW, respectively, with an equal number of uninfected PBMCs that had been stimulated with PHA and IL-2 for 48–72 hr. These standard cultures were maintained as per the AIDS clinical trials group (ACTG) consensus protocol at the Westmead Hospital. The cultures were maintained by two weekly passages with fresh PBMCs for at least 8–11 weeks. Co-cultures were routinely maintained at 3-day intervals for viral replication by the measurement of p24 antigen. Furthermore, the cultures were assayed for particle-associated reverse transcriptase (RT) activity and also for integrated proviral DNA copies by the polymerase chain reaction (PCR).

For the genetic analyses, DNA was extracted from fresh PBMCs (5×10^6 cells) using a QIA-Amp blood extraction kit (Quiagen, Germany) as per the manufacturer's instructions. One microgram of column-purified DNA was used for PCR with vpr-specific external primers (vpr-ext-1 5′TAACAGAAGATAGATGGAAC3′ with vpr int-2). The reaction with external primers was run for 25 cycles, and 5 ml of this was then subjected to further amplification with the internal primers (vpr-int-1 5′ATGGAACAAGCCCCAGAAGACCAGGGCC3′ and vpr-int-2 5′TAGGCTGACTTCCTGGATGCTTCTTCCAGGGCTCTA 3′) for 30 amplification cycles. The PCR conditions for both reactions were 94° for 3 min, 55° for 1 min; 72° for 2 min (1 cycle); 94° for 1 min; 55° for 30 sec; 72° for 2 min (with 25 and 30 cycles, respectively).

Extraction of RNA from plasma was done by subjecting plasma for virus pelleting by ultracentrifugation at 100,000 rpm for 1 hr. Viral pellets were resuspended in lysis buffer (5 *M* guanidium thiocyanate, sodium citrate, sarcosyl, 2-mercaptoethanol), phenol:chloroform extracted, and isopropanol precipitated (*32*). Following centrifugation at 15,000 rpm for 15 min, pellets were washed twice with 70% ethanol and dried, and the viral RNA was resuspended in 10 μl of DEPC-treated water. Ten milliliters of plasma-derived RNA was reverse transcribed for 1 hr at 42° in 50 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* spermidine, 10 m*M* DTT, 5 m*M* dNTPs, 5 pm/ml random hexamers (Boeringer Mannheim, Germany), 0.5 μl of RNAsin (Pharmacia, Sweden), 0.5 μl of AMV reverse transcriptase (Gibco BRL). For cDNA amplification, the aforementioned conditions were used with vpr external and internal primers.

The PBMC- and plasma-derived PCR products were run on 1% agarose (Promega). The gel was visualized under UV light and was subsequently transferred overnight by alkali denaturation onto a nylon membrane (Du Pont). The membrane was prehybridized for 2 hr at 42° and hybridized overnight at 42° by using [γ-^{32}P]ATP-labeled probe vpr det+ (5′ AGAAGACCAGGGGCCACAGAGGGAGCC 3′). The membrane was washed in 2× SSC and 0.1% SDS at 65° and autoradiographed overnight using Hyperfilm (Amersham, UK). For cloning, the PCR-amplified products were ligated into pGEM-T vector (Promega) as per the manufacturer's protocol. Fifty microliters of competent *Escherichia coli* cells (strain *JM109*) were transformed using 4 μl of ligation mix. The recombinants were plated on Luria broth–agar with ampicillin (75 mg/ml) containing 50 μl of X-gal (40 mg/ml) and 20 μl of IPTG (100 mg/ml), and incubated overnight at 37°. The recombinants were transferred onto nylon filters, followed by alkaline denaturation and neutralization, with subsequent hybridization with [γ-^{32}P]ATP-labeled vpr

probe. The positive clones were streaked onto the fresh plates for further purification and the single colonies were picked and cultured overnight at 37° in Luria broth with ampicillin at 37° under vigorous shaking. The bacterial cells were pelleted and the DNA was extracted as previously described (*33*). The purified vpr clones were sequenced with [α-^{35}S]dATP (Amersham, Australia) by the dideoxy chain-termination method using the Sequenase version 2.0 kit (Amersham-USB). The sequencing reactions were run on 6% Sequagel (National Diagnostics), and then the gel was dried and autoradiographed overnight.

Multiple sequence and peptide alignments were generated by using software available from the GCG package; the secondary structure predictions and hydropathicity profiles were generated by using the Chou and Fasman algorithm (*34*) and Kyte and Doolittle predictions (GCG package) (*35*), respectively. The average mass of Vpr protein was calculated by using software DNA 1.8 (Frederic Dardel, France). Phylogenetic relationships were studied using CLUSTAL (*36, 37*) and trees were bootstrapped with 1000 replications.

Coculture studies of fresh PBMCs from both mother and child showed that the mother and child were culture positive for HIV-1, and even after 8 weeks of culture no cytopathic effect was observed. These findings are consistent with other studies (*3, 9, 24*) in which no recoverable infectious virus was detectable in the cells from people who have survived with HIV-1 infection for $>$13 years. Although the mother and child harbor replication-competent strains of HIV-1, it is clear that the HIV-1 strains from both mother and child lack the ability for cellular permissiveness required for productive HIV-1 infection.

We could successfully amplify the 291-bp vpr fragment from the PBMCs (collected in 1987, 1989, 1991, 1993, 1994, and 1995) (Fig. 1A) and plasma (collected in 1994 and 1995) (Fig. 1B) of both mother and child. Sequencing of vpr revealed that most changes were clustered in the basic amino acid-rich domain (aa residues 83–96), which was most susceptible to in-frame insertions, deletions, out-of-frame deletions, and nonconservative base substitutions (10). These changes resulted in length polymorphism in the vpr gene in both PBMC- and plasma-derived vpr clones, and as a consequence, average molecular mass of Vpr protein was affected. Although the amino acid changes that are distributed over the entire length of the Vpr protein sequence before aa residue 83, they do not have the potential to modulate the length polymorphism and molecular mass of the Vpr protein (Figs. 2A and 2B). The most commonly reported Vpr length is 96 aa. However, there are two HIV-1 sequences described with length polymorphism in the vpr gene (*38*). Only HIV-1 MPV5180 has a 9-bp insertion, which results in 3 additional amino acids (SNT) being included in the protein, and it belongs to subtype O (based on the vpr gene). The changes in HIV-1 MPV5180 occur at the same position where we have observed insertions, deletions, or base substitutions in the HIV-1 strains. Analysis of these HIV-1 strains in the V3 region has previously indicated that they belong to subtype B, and minimal length polymorphism is seen in the vpr gene of subtype B HIV-1 isolates (*38, 39*).

In addition, we have also analyzed HIV-1 vpr gene peptide sequences directly from PBMCs of 30 other patients (which includes male and female patients, nontransmitting and transmitting mothers and children) who died of AIDS or have AIDS-related illnesses. Comparison of the C-terminal Vpr sequences clearly suggested that HIV-1 strains derived from these patients neither have any abnormality nor do they exhibit length polymorphism (Fig. 2D), as observed in the DNA and RNA clones derived from both long-surviving HIV-1 infected mother (LW) and child (JW). So far, we have seen C-terminal mutations only in 3 HIV-1 infected intravenous drug users, but a detailed analysis viral quasispecies did not show predominance of such mutations *in vivo* (*10*).

Peptide sequence comparisons of the clones derived from PBMCs and plasma of both mother (LW) and child (JW) indicated that a compartmentalization occurred between PBMC and plasma-derived vpr clones. Interestingly, a major difference between the PBMC- (1987, 1989, 1991, 1993, 1994, and 1995) (Fig. 2A) and plasma- (1994 and 1995) derived vpr clones (Fig. 2B) is the occurrence of fewer defects at the C-terminus region of the Vpr protein in plasma with the peptide length of 95 amino acids caused by an in-frame deletion of an arginine (R-88). Also, the plasma-derived clones are more homogeneous as regards the genetic variation in their vpr genes. The only unique changes in plasma RNA clones was a deletion of alanine at position 89 in 9 of 13 clones. In contrast, the PBMC-derived vpr clones showed a greater variation at the C-terminus, with in-frame insertions or deletions causing considerable length polymorphism in the Vpr protein. In PBMCs, the Vpr peptide length ranged from 95 to 98 aa. Interestingly, in 1987 a PBMC-derived Vpr clone from the mother (lw87-1b) had a 5-bp out-of-frame insertion (Fig. 1A). Also, several signature changes, between PBMCs and plasma-derived clones, were also identified at amino acid residues 28, 37, 41, 55, 77, and 83 (Figs. 2A and 2B).

Dissection of the RNA and DNA clones by phylogenetic analysis suggests that both vpr plasma RNA and PBMC DNA clones are not related in time. Additionally, the analysis of 13 vpr RNA clones in 1994 and 1995 from both mother and child showed separate branching patterns, along with the intermingling of 1994 and 1995 vpr clones (data not shown).

The vpr clones derived from PBMC DNA of both mother and child from 1987, 1989, 1991, 1993, 1994, and 1995 indicated a more complex pattern of divergence between clones derived from different years. The interspersed pat-

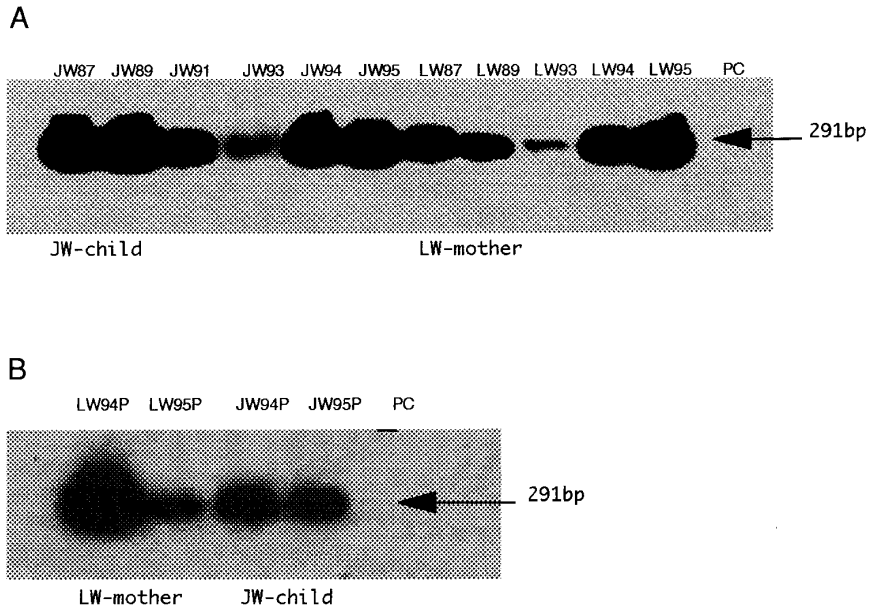


FIG. 1. (A) The hybridization of vpr gene PCR products derived from PBMCs of mother (LW) and child (JW). The products were hybridized with a [γ-^{32}P]dATP labeled Vpr det+ probe. PC is primer control. (B) The hybridization of vpr gene PCR products derived from plasma of mother (LW) and child (JW). The products were hybridized with [γ-^{32}P]ATP-labeled vpr det+ probe. PC is primer control.

tern of PBMC-derived clones on the phylogenetic tree further suggested a strong interrelationship between various vpr clones and the HIV-1 strains from both mother and child (data not shown). A more pronounced pattern of intermixing of PBMC-derived clones could be due to the evolution of the same viral quasispecies *in vivo* from 1987 to 1985, which was transmitted from mother to child.

Combined analysis of RNA and DNA vpr clones indicated an interesting pattern of segregation between plasma- (collected in 1994 and 1995) and PBMC- (collected in 1987, 1989, 1991, 1993, 1994, and 1995) derived clones. Interestingly, the plasma-derived clones were more genetically related to the PBMC-derived clones only in 1991, 1993, 1994, and 1995, whereas the PBMC vpr clones derived in 1987 and 1989 diverged earlier (Fig. 3). This may be due to greater diversity in earlier PBMC DNA clones, but we do not have plasma RNA clones to validate this.

In this HIV-1-infected nonprogressing mother–child pair, we could find a definitive correlation between transmission and genetic homogeneity in the vpr. This is in contrast to the V3 region, which undergoes selection, and previously no correlation was found between degree of heterogeneity in V3 and transmission strains between mother and child (*40*).

In some cases the HIV-1 RNA population of the mother was more related to the HIV-1 RNA population of the child, whereas in other cases it was more related to the virus DNA population (Fig. 2A), suggesting that either cell-free or cell-associated virus may have been transmitted. Based on this genetic relationship between plasma RNA and PBMC DNA vpr clones from both mother and child, it is likely that the source of virus in plasma may be a subset of transcriptionally active CD4+ lymphocytes, or virus could be secreted into the circulation by cells sequestered in solid tissue. Previously, it had been shown that plasma from both asymptomatic and symptomatic individuals is infectious (*41*) and that infection of PBMCs may be a self-sustaining process. Infection, and continued sequence evolution of HIV, may indeed take place in peripheral CD4+ lymphocytes. A similar trend of *in vivo* evolution of vpr quasispecies was also evident from our analyses.

Secondary structure predictions of the amino acid residues (77–96) at the C-terminus of Vpr, using the Chou and Fasman algorithm (*34*), also suggested that the naturally occurring mutations (insertions, deletions, and substitutions) between aa residues 83 and 89 have a profound effect on the secondary structure of Vpr protein (Fig. 4) (*10*). Mutational changes mostly affected β-turns, which are crucial in structural stabilization of the protein. Furthermore, our peptide comparisons suggest that 62% of PBMC-derived clones showed an introduction of a proline at residues 85 and 87, replacing the highly charged arginines at these positions. In contrast, 69% of plasma-derived clones showed the occurrence of the introduction of prolines at positions 85 and 87 (Fig. 2B). It will be recalled that the protein linkages of the nitrogen atom in proline consists of imido groups (—CO—N═) instead of amino groups (—CO—NH═) formed by all other amino acids. The nitrogen group in proline is thus unable to form the crucial hydrogen bond, having no hydrogen available for the purpose. As a result, proline will mark a sudden bend in the axis of the coil (*42*).

A

	aa 1-16 N-terminal Domain	aa 17-34 a-Helical Domain I	aa 35-45 Loop Region	aa 46-74 a-Helical Domain II	aa75-76 DPM	aa 77-96 C-terminal domain	Length
				# #	# ++	#	
PNL43	MEQAPEDQGPQREPYN	EWTLELLEELKSEAVRHF	PRIWLHNLGQH	IYETYGDTWAGVEAIIRILQQLLFIHFRI	GC	RHSRIGVTRQRR--ARNGASRS*	96aa
JW87-1		A N	P S	T T		Q I -- *	96aa
JW87-2		D T	S	T T		Q I -- *	96aa
JW87-3		A N	P S	T T		Q I -- *	96aa
JW89-1		A	G			IIP -- *	96aa
JW89-2		A	G			IIP -- *	96aa
JW89-3		A I	G			IIP -- *	96aa
JW89-4		A I	G			IIP -- *	96aa
JW89-5		A I	G			IIP -- *	96aa
JW91-2		K	L S R	K T L		Q I PR --- R *	95aa
JW91-3		K	L S R	T	R	I - --T *	95aa
JW91-4	D	A K	L S	T L		Q IIPR P--- *	95aa
JW91-5	D	A K	L S	T L		Q IIPR P--- *	95aa
JW93-1		N K	L S Y	T L		Q IIPR P--- *	95aa
JW93-2		A N R	S S	T L		Q IIPR P--- *	95aa
JW93-3		A N K	L S	T L		Q I PR P--- *	95aa
JW93-4		V N K	L S	T L		Q I PR P--- *	95aa
JW93-5		N K	L S	T L		Q I PR P--- *	95aa
JW94-1		N K	L S R	T L		Q PR P--- *	95aa
JW94-2		N K	L S	T L M T	R	Q IIPR P--- *	95aa
JW94-3		A N K	L S	T L		Q I PR P--- *	95aa
JW94-4		A N K	L S	T L M		Q PR P--- *	95aa
JW94-5		A N	P S	T L		Q I PR P--- *	95aa
JW95-1		N K	L S	T L M		Q I PRWPK--- *	95aa
JW95-4		A N K	L S	T L I		Q I PR P--- *	95aa
JW95-5		R N K	L S	T L M		Q I PR P--- *	95aa
LW87-1		A D T K	S L			Q M IIRFNGGEQEMEPVDP	
LW87-2		A D N	S			Q I P R- *	97aa
LW87-4		A D N	S			Q I P R- *	97aa
LW87-5		A D T	S Y			Q IIQTQ RG *	98aa
LW89-1		A D T D	S	T		Q I P R- *	97aa
LW89-2		A D T	S	T		Q I P R- *	97aa
LW89-3		A D T	S			Q I P Q R- *	97aa
LW89-4		A D T	S			Q I P Q R- *	97aa
LW89-5		A D T	S			Q T P R- G *	97aa
LW93-1	D	A N K	P S Y	T L		Q IIPRRP--- *	95aa
LW93-2		A N K	P S Y	T L		Q IIPRRP--- *	95aa
LW93-3	D	N K	L S	T L		Q IIPR P--- C *	95aa
LW93-4		N K	L S	T L R		Q IIPR P--- C *	95aa
LW94-2		N	P S	T GV		Q I -- *	96aa
LW94-3		N R	P S	T GV T		-- *	96aa
LW94-4		N	P S	T GVV T		Q I -- *	96aa
LW94-5		N	P S	T GV T		Q I -- *	96aa
LW95-1		NG	P S	T N		Q II -- *	96aa
LW95-2		D T	S Y	T		Q I -- *	96aa
LW95-3		N	P S	T T		Q I -- *	96aa
LW95-4		N	P S	T T		Q I -- *	96aa
LW95-3'		N	P S	T		Q II -- *	96aa
LW95-4'		N	P S T	T V		Q II -- *	96aa
LW95-5'		N	P S	T V		Q II -- *	96aa

B

	aa 1-16 N-terminal Domain	aa 17-34 a-Helical Domain I	aa 35-45 Loop Region	aa 46-74 a-Helical Domain II	aa75-76 DPM	aa 77-96 C-terminal domain	Length
				# #	# ++	#	
PNL43 VPR	MEQAPEDQGPQREPYN	EWTLELLEELKSEAVRHF	PRIWLHNLGQH	IYETYGDTWAGVEAIIRILQQLLFIHFRI	GC	RHSRIGVTRQRRARNGASRS*	96aa
JW94-2P VPR		N K	L S	T		Q I PR P- *	95aa
JW94-3P VPR		A K	L S	T L T		Q I PR P- *	95aa
Jw94-4P VPR		N K	L S	T		Q I PR P- *	95aa
JW95-1P VPR		A NV K	L S	T L M		Q IIPR P- *	95aa
JW95-2P VPR		A N K	L S	T L M		Q IIPR P- *	95aa
JW95-3P VPR		N K	L S	T L M		Q IIPR P- *	95aa
LW94-1P VPR		A K	L S	T L		Q I PR P- *	95aa
LW94-2P VPR		A K	L S	T L		Q I PR P- *	95aa
LW94-3P VPR		A N K	L S F	T L		Q I PR P- *	95aa
LW95-1P VPR	Q	H R	P S	T T		Q I *	96aa
LW95-3P VPR		N	SL S	T V		Q II *	96aa
LW95-6P VPR		N	SL S	T T		Q I *	96aa
LW95-7P VPR		H	P S	T T		Q I *	96aa

C

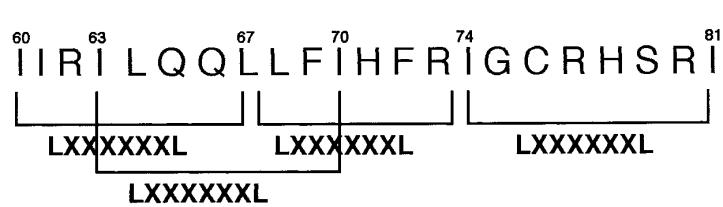


FIG. 2. The peptide alignment of PBMC-derived clones of the vpr gene. Boxed areas are various functional domains within the Vpr protein. DPM is a dipeptide motif; # indicates residues participating in leucine zipper formation; - - - indicates deletions; * denotes stop codon; and pNL43 is the HIV-1 prototype sequence. (B) The peptide alignment of plasma-derived RNA clones of the Vpr protein. (C) Peptide sequence of Vpr showing four leucine zipper motifs (LZM) (LxxxxxxL) located between amino acid residues 53 and 74. Leucine or isoleucine participates in LZM formation. (D) C-terminus (aa 81–96) peptide alignment of 30 HIV-1 Vpr sequences derived from the PBMCs of patients who died of AIDS-related illnesses or who have AIDS. For genetic comparisons of the C-terminus sequences of the Vpr peptide sequence, we have chosen three HIV-1 infected groups: (i) male and female patients, (ii) mother and child pairs, and (iii) nontransmitting mothers, respectively.

D

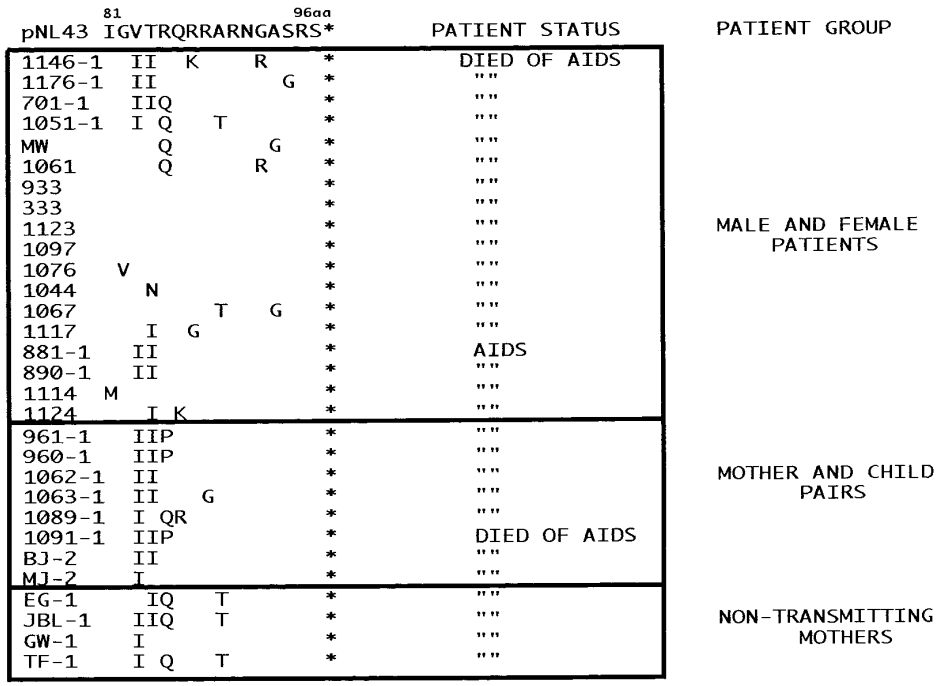
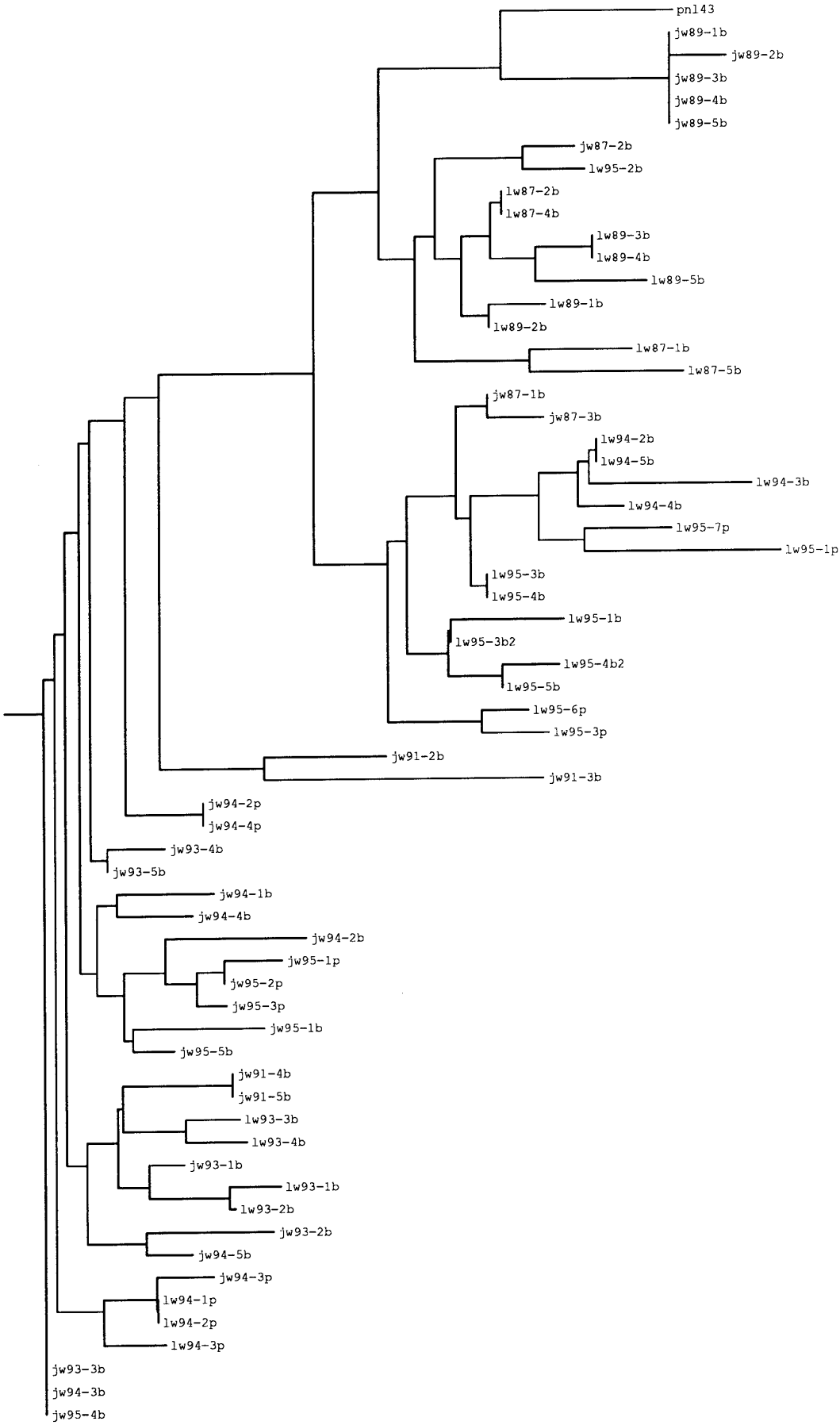


FIG. 2—*Continued*

Previously (*42*) proline rings have been found at places in the hemoglobin molecule where the chain changes direction and abandons the helix (*43*). Interestingly, the clones which did not show the introduction of prolines revealed amino acid substitutions at position 70, which is crucial in the formation of leucine zipper motif (LxxxxxxL) (Fig. 2C). This naturally occurring mutation (residue 70 I → V or T) alters the zipper located between residues 60 and 81 from ILQQLLFI → ILQQLLFT or V (Fig. 2A). Leucine zipper motifs (LZM), which are formed by the participation of both leucine and isoleucine residues, are found commonly in DNA-binding proteins or transcriptional activators (28). Usually, leucine zipper motifs reveal a tight evolutionary conservation in the vpr gene of the previously reported HIV-1 isolates/subtypes (*38*). Previously, it has been suggested that the leucine/isoleucine-rich domain (aa 60–75) may impart to Vpr a weak transcriptional activity (21). Moreover, Vpr is regarded as a weak transcriptional activator, and any mutations in the leucines or isoleucines abolish the functional activity of Vpr (*12*).

In the light of a recent evidence regarding the mutations in the vpr gene can cause chronic HIV-1 infection (*18*), and also the role of C-terminal amino acid residues (aa 83–90) in G2 cell cycle arrest during HIV-1 infection, it is likely that these mutations occurring between residues 83 and 89 may, in part, be responsible for chronic infection in this mother–child pair, which has survived for >13 years with stable CD4+ T-cell counts. Since Vpr protein is also known to participate in productive human macrophage infection (*44–46*), it may be that these naturally occurring mutations at the 3′ end of the vpr gene of HIV-1 derived from both mother and child may have relevance in the chronicity of HIV-1 infection. Furthermore, a comparison of Vpr C-terminal sequences of HIV-1 strains derived from 30 different patients (including male and female patients, mother–child pairs, and non-HIV transmitting mothers, who either died of AIDS-related illnesses or have AIDS) lends further credence to the importance and specificity of these changes at the C-terminus of the vpr gene derived from the long-surviving mother–child pair.

Our *in vitro* culture studies suggest that the viral strains from both mother and child transiently infected T-cells, and they lack the ability for productive HIV-1 infection. Furthermore, the cells that have survived infection with HIV may therefore be due to highly selected subset of the infecting virus strains (originating in the course of evolution of viral quasispecies *in vivo*), whose noncytopathic properties ensure the long-term survival of this mother–child pair. These infecting strains are predominant *in vivo* (>62%) and show defects at the C-terminal region of the Vpr, along with enormous polymorphism in the length of Vpr resulting from these mutations at the C-terminal end in both PBMC- and plasma-derived clones. Recently, it has been suggested that for HIV-1 pathogenesis *in vivo,* the ratio and/or the balance between the defective and nondefective clones may play an important part. This is consistent with previous studies (*8*) which suggest that 60% defective HIV-1 clones in the acces-


pn143
jw89-1b
jw89-2b
jw89-3b
jw89-4b
jw89-5b
jw87-2b
lw95-2b
lw87-2b
lw87-4b
lw89-3b
lw89-4b
lw89-5b
lw89-1b
lw89-2b
lw87-1b
lw87-5b
jw87-1b
jw87-3b
lw94-2b
lw94-5b
lw94-3b
lw94-4b
lw95-7p
lw95-1p
lw95-3b
lw95-4b
lw95-1b
lw95-3b2
lw95-4b2
lw95-5b
lw95-6p
lw95-3p
jw91-2b
jw91-3b
jw94-2p
jw94-4p
jw93-4b
jw93-5b
jw94-1b
jw94-4b
jw94-2b
jw95-1p
jw95-2p
jw95-3p
jw95-1b
jw95-5b
jw91-4b
jw91-5b
lw93-3b
lw93-4b
jw93-1b
lw93-1b
lw93-2b
jw93-2b
jw94-5b
jw94-3p
lw94-1p
lw94-2p
lw94-3p
jw93-3b
jw94-3b
jw95-4b

aa	81 82 83 84 85	86 87 88	89 90 91 92 93 94 95 96	
PNL43	I(B)G(B)V(B)T(B)R(B)-	Q(•)R(•)R(•)-	A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	96aa
JW87-1b	I(•)G(•)I(•)T(•)R(•)-	Q(•)R(•)R(•)-	A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	96aa
JW89-1b	I(B)G(B)I(B)I(B)P(•)-	Q(t)R(t)R(•)-	A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	96aa
JW91-3b	I(b)G(b)I(b)T(b)Q(b)-	- R(b)R(b)-	T(b)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	95aa
JW93-1b	I(B)G(B)I(B)I(B)P(•)-	- R(•)R(•)-	P(T)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	95aa
JW94-1b	I(B)G(B)V(B)T(B)P(T)-	- R(T)R(•)-	P(T)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	95aa
JW95-1b	I(B)G(B)I(B)T(B)P(T)-	- R(T)W(•)-	P(T)K(T)N(T)G(•)A(t)S(t)R(t)S(•)	95aa
JW95-2p	I(B)G(B)I(B)I(B)P(•)-	- R(•)R(•)-	P(T)R(T)N(T)G(T)S(T)S(T)R(T)S(•)	95aa
LW87-2b	I(B)G(B)I(B)T(B)P(t)-	Q(t)R(t)R(•)R(•)A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)		97aa
LW87-5b	I(B)G(B)I(B)I(B)Q(B)T(•)Q(•)R(t)R(t)G(t)A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)			98aa
LW89-3b	I(•)G(•)I(•)T(•)P(•)Q(h)Q(h)R(h)R(h)-	A(h)R(h)N(•)G(•)A(t)S(t)R(t)S(•)		97aa
LW93-1b	I(B)G(B)I(B)I(B)P(•)-	- R(•)R(•)-	P(T)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	95aa
LW95-1b	I(B)G(B)I(B)I(B)R(B)-	Q(•)R(•)R(•)-	A(•)R(T)N(T)G(•)A(t)S(t)R(t)S(•)	96aa
LW95-6p	I(•)G(•)I(•)T(•)R(•)-	Q(•)R(•)R(•)-	A(•)R(T)N(T)G(T)S(T)S(T)R(T)S(•)	96aa

FIG. 4. The secondary structure predictions based on the defects clustered at the C-terminus of the vpr gene. Only basic amino acid-rich region (aa 81–96) was analyzed. B, β-sheet; b, propensity for β-sheet; T, β-turn; t, propensity for β-turn; h, propensity for α-helix. (*) is neutral change. pNL43 is the HIV-1 prototypic isolate. The far right end shows the total Vpr peptide length in each case.

sory/regulatory genes (vif, vpr, nef, vpu, and tat) play an important part in conferring long-term protection in LTNPs.

Interestingly, our previous work on the HIV-1 nef gene from both mother and child suggested that 80% population of nef gene population was open in PBMC-derived clones, but prematurely terminated by only one amino acid. The remaining 20% of the total DNA clones showed random deletions in the nef gene ranging between 200 and 350 bp (*9*). In contrast, 100% RNA clones were open but they too terminated prematurely by one amino acid. In both cases, terminal cysteine (cys-206) was eliminated by an ochre stop codon (*9*). These data further suggest that the most likely scenario for the long-term survival of this mother–child pair with chronic HIV-1 infection could be due to predominance of defects in the vpr gene quasispecies derived from in both plasma and blood. Thus, deletions in the nef gene may not be the only viral factor which can confer protection in HIV-infected humans or SIV-infected monkeys (3, 6). It is likely that defects in other regulatory genes may have a similar impact on viral replication and infectivity.

Although the significance of C-terminus of Vpr in G2 cell cycle arrest as a step to early HIV-1 infection has recently been described (*18*), the role of these naturally occurring C-terminus mutations in long-term HIV-1 infection remains obscure. At this time, we cannot explain the exact role of vpr length polymorphism in modulating HIV-1 pathogenesis in this long-surviving mother–child pair. There are several hypotheses which emerge from these data. (i) It is likely that vpr length polymorphism, owing to mutations at the C-terminal region, may be a characteristic of viral quasispecies in this subset of patients; (ii) it may be that these mutations are not allowing the productive viral infection by down-regulating viral replication *in vivo;* and (iii) since the N-terminal region of tat gene lies at the C-terminal region of the vpr gene, it is likely that mutations (in some cases) may further affect the tat gene open reading frame. It should be recalled that Tat is a transcriptional co-activator and plays a vital role in regulating translation (*47*).

These are the first analyses of the vpr gene in a long-surviving genetically linked mother–child pair, and they suggest an avenue for further study. No reports have defined the biological role of these mutations in G2 cell cycle arrest by HIV-1; only *in vitro* analyses could explain if these mutations really form the basis for chronic HIV-1 in this mother–child pair. In summary, these studies will continue to provide deeper insights into molecular mechanisms of HIV-1 pathogenesis *in vivo.*

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FIG. 3. A combined phylogenetic analysis of PBMC- (b) and plasma- (p) derived Vpr clones from both mother and child. CLUSTAL was used for alignment, and PHYLIP was used for constructing phylogenetic tree. Tree was bootstrapped with 1000 replications, and each branch length on tree has 80–100% confidence value. pNL43 is the prototypic HIV-1 sequence used for comparisons.

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